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Assessing the relationship between tumor-infiltrating lymphocytes and PD-L1 expression in triple negative breast cancer: Identifying optimal TILs cut-off value for pathologic reporting

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A R T I C L E I N F O

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A B S T R A C T

Background: Triple Negative Breast Cancer (TNBC) presents diagnostic complexities, particularly in evaluating Tumor-Infiltrating Lymphocytes (TILs) and Programmed Death-Ligand 1 (PD-L1) expression. This study aimed to identify optimal TILs percentage cut-offs predictive of PD-L1 expression and to investigate the relationship between TILs, PD-L1, and tertiary lymphoid structures (TLSs).

Method: Method: Analyzing 141 TNBC cases, we assessed TILs, PD-L1 expression (clones 22C3 and SP142), and TLS presence.

Results: We identified TILs cut-offs (<20 %, 20–60 %, ≥60 %) correlating with PD-L1 expression. TILs <20 % rarely express PD-L1 with either 22C3 or SP142 clones. TILs ≥60 % demonstrate PD-L1 expression across both clones. TILs within the 20–60 % range correlate with PD-L1 expression using the SP142 clone, but not 22C3. Evaluating TILs solely at the tumor edge led to inaccuracies, highlighting the need for overall assessment of TILs throughout the entire lesion. TLS presence correlated with higher TIL percentages and PD-L1 expression, particularly with SP142. Discrepancies between 22C3 and SP142 clones (15 % vs. 50 % positivity, respectively) underscored the variability in PD-L1 detection.

Conclusion: This study identifies TILs cut-offs predictive of PD-L1 positivity, suggesting the need for institutions to tailor these thresholds based on the selected PD-L1 clone and treatment. Evaluating TILs solely at the tumor edge may overlook the complexity of tumor immune infiltration. While TLS presence correlates with higher PD-L1 expression, particularly with the SP142 clone, its exact predictive value for PD-L1 remains to be clarified. The SP142 clone exhibits higher positivity rates compared to 22C3.

1. Introduction

Tumor-infiltrating lymphocytes (TILs) have emerged as an important aspect of the immune response within the tumor microenvironment, especially in the context of the triple-negative breast cancer (TNBC) subtype. TILs interact with various cells, shaping a unique microenvironment that affects.

disease progression and treatment outcomes [1]. The number of TILs presents within the tumor has been identified as a critical factor affecting patient prognosis, survival rates, and response to treatment [2,4,5]. TNBC has a higher TIL count compared to other subtypes. Denkert et al.'s study [6], along with other studies [3,4], indicates that elevated TIL levels are linked to a reduced risk of recurrence and extended disease-free survival.

In 2014, the International TILs Working Group, a global collaboration involving universities and hospitals, introduced a standardized method for evaluating TILs on H&E slides to ensure consistency in evaluation [7].Assessment of PD-L1 levels can be performed using visual histologic intensity scores, and correlating these scores with results from digital image analysis showed a strong and consistent correlation.

However, limitations in the reporting format persist. A key issue is the absence of clear guidelines for reporting TILs quantity, such as percentage range, low-intermediate-high categories, along with a lack of defined threshold values. Unlike hormonal status tests, the ambiguity in reporting TILs parameters undermines the utility of investing time in TILs assessment in pathology reports for guiding patient treatment

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decisions.

In TNBC, PD-L1 expression, alongside TILs, plays a complex role in shaping the tumor microenvironment [12,13,16-18]. Tumors with PD-L1 expression often exhibit a higher presence of PD-L1-positive lymphocytes, which frequently aligns with higher TILs levels, impacting clinical outcomes, leading to a more favorable prognosis, and influencing treatment response [10,13,16,17,19,25]. This expression is attributed to oncogenic processes within the tumor or elevated interferon-γ production by TILs, which acts as a negative immune regulator, enhancing tumor PD-L1 expression. As a result, even when there's a high number of immune cells, they might not effectively eliminate tumor cells due to the persistent PD-L1 expression. PD-L1 inhibitors, such as Pembrolizumab (KEYTRUDA) and Atezolizumab (TECENTRIQ), along with specific PD-L1 immunohistochemistry companion clones like 22C3 and SP142, have shown significant survival benefits in TNBC patients in clinical trials [23,24,33]. To this end, we determined the TILs threshold in TNBC that indicates a strong likelihood of PD-L1 expression. By pinpointing this optimal cut-off, which implies substantial PD-L1 expression, TILs surpassing this threshold not only acts as a positive predictive indicator for the patient but also guides clinicians on when a PD-L1 immunohistochemical study might be warranted for that case.

2. Materials and methods

2.1. Ethical statements

Our study received approval from the Institutional Review Board at the Institute of Pathology (IOP, No. IOP-KMR64-002), which granted a waiver for the documentation of informed consent due to the non-involvement of human subjects. This approval confirms our adherence to ethical standards in research.

2.2. Case selection: triple-negative breast cancer resection specimens

We analyzed a cohort of 173 triple-negative cases, which comprise the mastectomy specimens sent for tissue processing at the Institute of Pathology (IOP) in Bangkok, Thailand, between 2020 and 2021. Following a comprehensive histological review, ancillary tests, including estrogen receptor (ER), progesterone receptor (PR), and HER2 immunohistochemical studies, were also reviewed. We include cases that were definitively negative for estrogen receptor (ER), progesterone receptor (PR), and HER2. For estrogen and progesterone receptor testing, cases with <1 % immunoreactive tumor cells are considered negative. HER2 scores of 0 and 1+ are considered negative. For every instance presenting an equivocal HER2 score of 2+, dual in situ hybridization (DISH) studies were reviewed, and cases with positive DISH results were excluded. These assessments were held by two experienced pathologists from IOP and each possessing a minimum of two years of experience. Following the assessments, 32 cases were subsequently excluded from the study. Some of them were excluded because they exhibited low positive ER results (1 to 10 % of tumor cells staining for ER), which did not meet the inclusion criteria. A few excluded cases lacked a DISH study, despite the HER2 result being equivocal. The remaining cases were excluded because the paraffin blocks could not be obtained or retrieved. Consequently, our study included 141 cases that underwent detailed TILs evaluation and PD-L1 examinations.

2.3. Tumor infiltrating lymphocytes (TILs) evaluation

The TILs assessment at IOP was carried out by two experienced pathologists in adherence to the 2014 guidelines set by the International TILs Working Group. They independently and systematically examined each slide, selecting one section believed to best represent the tumor lesion with the most pronounced intratumoral inflammation. If their results do not differ by >10 %, the scores will be averaged. If there is a difference >10 %, the case will be jointly reviewed, and a consensus percentage will be determined.

2.3.1. Selected tumor area

We evaluated one H&E section for each of the 141 cases, covering the entire tumor on the chosen slides instead of just hot spots. We began with low magnification (x40) to examine invasive tumor regions, then used medium to high magnification (x200 to x400) for stromal areas and TILs percentage estimation. Detailed assessments, like differentiating infiltrated cell types, were done at high power (x400).

2.3.2. Tumoral TILs vs stromal TILs

Tumoral TILs are lymphocytes and plasma cells within tumor nests, while stromal TILs are found in the stroma between tumor cells. Both are considered "True TILs" since they reside within tumor areas. Historically, intratumoral TILs were thought to be more clinically significant than stromal TILs. However, recent research suggests that this distinction might be artificial, as cells can migrate between the two areas [7]. Stromal TILs are now favored for evaluation due to their consistency across studies [3,7,9,20,21]. Intratumoral TILs vary more, making assessment difficult without specialized studies, whereas stromal TILs provide a more uniform representation regardless of tumor characteristics. Hence, our study exclusively focuses on evaluating stromal TILs.

2.4. Selection criteria for stromal TILs, invasive edge, and tertiary lymphoid structures

Stromal TILs were assessed within the boundaries of the invasive tumor, inclusive of the invasive edge. Stromal areas around DCIS or adjacent normal lobules were excluded. We also separately assessed TILs at the invasive edge, a stromal region extending up to 1 mm from the invasive boundary. The biology and density of TILs here might differ from those within the tumor. Given the unclear data on this area, evaluating the invasive edge separately from the inner stroma might benefit future research. Occasionally, robust immune responses at this edge result in follicular lymphoid aggregations, potentially with germinal centers, termed tertiary lymphoid structures (Fig. 2). These aren't included in the standard TILs count but are documented separately. We omitted areas with artifacts, necrosis, severe inflammation, hemorrhage, histiocytic aggregation, biopsy sites, or significant fibrosis. Of particular significance, stroma with dense collagen is carefully differentiated from hyalinization/fibrosis and is included in the evaluation area. TILs percentages, based on mononuclear cells, were documented as semiquantitative data, excluding macrophages, histiocytes, dendritic cells, granulocytes, and polymorphonuclear leukocytes. Immunohistochemical studies didn't offer advantages over conventional H&E assessment [7]. For tumor foci <5 mm apart, intervening stroma was included, as they likely represent extensions of the main carcinoma [34]. If over 5 mm apart, TILs were assessed separately for each focus and then averaged.

2.5. Evaluating stromal TILs percentage

We computed stromal TILs as the proportion of the stromal area occupied by mononuclear inflammatory cells relative to the entire intratumoral stromal area. Instead of counting stromal cells or nuclei, we considered the dispersed nature of lymphocytes, which typically disperse rather than form densely solid aggregates. Thus, even with high stromal TILs percentages like 90–100 %, individual lymphocytes remain separated by small spaces. Our methodology adhered to the guidelines of the International TILs Working Group 2014 [7] (Fig. 3). Furthermore, we referred to the comprehensive photo resources available on www.tilsinbreastcancer.org and materials provided by the TILs international work group to illustrate TILs percentages. To ensure consistency and clarity in reporting, we presented TILs percentages in 10 % increments, including 0 %, 1 %, 5 %, 10 %, 20 %, and so forth, up to 100 %. Our evaluation was performed at x200 magnification to assess the TILs

percentages systematically across all tumor areas. From each TILs percentage parameter within each field, we subsequently determined the average value.

2.6. PD-L1 immunohistochemical studies

In our examination of whole sections from 141 TNBC cases, we utilized the FDA-approved PD-L1 clones 22C3 (DAKO PD-L1 IHC 22C3, EnVision FLEX) at Ramathibodi Hospital, Bangkok and SP142 (VENTANA PD-L1 IHC SP142, OptiView DAB) at the Institute of Pathology, Bangkok. Both clones were applied using standard protocols as a companion tests: 22C3 for Pembrolizumab (KEYTRUDA) and SP142 for Atezolizumab (TECENTRIQ). Scoring was conducted by two pathologists who are board-certified by the Thai Board of Pathology and have received specific training in PD-L1 22C3 and SP142 CDx scoring for TNBC. For PD-L1 22C3 [23,26], at least 100 tumor cells were assessed using the Combined Positive Score (CPS) method, which divides PD-L1 staining cells (tumor cells, lymphocytes, macrophages) by total viable tumor cells, multiplied by 100. A CPS of 10 or more indicated PD-L1 positivity. For PD-L1 SP142 [24,27], a minimum of 100 tumor cells were evaluated based on tumor-infiltrating immune cells (IC) score. An IC score of 1 % or more marked a positive case. Two pathologists independently assessed both PD-L1 clones. If their results differ, borderline cases undergo joint analysis to reach a consensus.

2.7. Statistical analysis

Data were quantitatively analyzed using descriptive statistics, specifically frequency counts, via IBM SPSS Statistics.

26. The optimal cut-off for TILs in TNBC was determined in relation to PD-L1 positivity using the Receiver Operating Characteristic Curve (ROC). Concordance between two PD-L1 clones was measured with the Cohen's kappa coefficient. Differences in TILs, TILs Infiltrative Edge, and TLS were assessed using nonparametric statistics in Program R. Results were deemed significant at a p-value of <0.05.

3. Results

3.1. TILs percentage, TILs exclusively at the invasive edge, and presence of tertiary lymphoid structures

In TNBC cases, the TILs percentage spans every 10 % increment, ranging from 0 to 100 %. Of these, 12.8 % have TILs <1 %, more than half of cases (67.4 %) have TILs exceeding 10 %, and 9.2 % surpass 80 % TILs (Table 1). Interestingly, the percentage of TILs present solely at the invasive edge is not significantly different compared to intralesional TILs, which combine both entire intratumoral TILs and those at the invasive edge (Table 1). Additionally, tertiary lymphoid structures (TLS) were identified in 15.6 % (22 out of 141) of the cases.

3.2. PD-L1 expression status

The clone 22C3 tested positive in 21 out of a total of 141 cases (14.9 %), as indicated by a CPS of ≥10. Meanwhile, half of the TNBC cases tested positive with SP142 (50.4 %), marked as positive when having an IC scare of ≥1 % (Table 2). There were no discrepancies in scoring between the pathologists.

3.3. Determining the optimal TILs percentage cut-off for PD-L1 expression and a comparative analysis between total intralesional stromal TILs and TILs exclusively in infiltrative edge areas

We aimed to identify the optimal cut-off value of the TILs percentage closely associated with positive PD-L1 expression. This was assessed for two distinct FDA-approved PD-L1 clones, as depicted in Table 3 and Fig. 1. For the clone 22C3, the proposed cut-off stands at ≥60 %, delivering a sensitivity of 95.2 % and a specificity of 84.2 %. For the clone SP142, the proposed cut-off is ≥20 %, with a sensitivity and specificity of 98.6 % and 84.3 %, respectively.

We further studied TILs localized at the tumor's invasive edge, comparing them to the entire intralesional stromal TILs. For the clone 22C3, the optimal cut-off at the infiltrative edge is ≥50 %, resulting in 95.2 % sensitivity but only 70 % specificity. In contrast, for clone SP142, the cut-off is ≥10 % with a sensitivity of 98.6 % but a lower specificity at 54.3 % (Table 4 and Fig. 1).

Comparatively, while both methods show high sensitivity, TILs at the Infiltrative Edge have a lower specificity than the overall intralesional TILs. Specifically, for the clone SP142, overall TILs demonstrated better diagnostic accuracy with an AUC of 0.966, compared to 0.906 for the Infiltrative Edge TILs, as validated by the ROC curve (see Table 5 and Fig. 1). Meanwhile, for the clone 22C3, the Infiltrative Edge TILs had a notably higher false positive rate, despite similar AUC values with the overall TILs. For the clone 22C3, while there is no statistically significant difference in overall diagnostic performance (AUC) between TILs and TILs Infiltrative Edge ($Z = 1.664$, P-value $= 0.1$), the Infiltrative Edge TILs exhibited a higher false positive rate compared to the overall TILs.

3.4. Utilizing TILs percentage thresholds to enhance prediction of PD-L1 expression status

The expression of PD-L1, particularly in the SP142 clone, becomes notably significant when the percentage of Tumor-Infiltrating Lymphocytes (TILs) is at or above 20 %. This metric serves as a potential indicator for the presence or absence of PD-L1 expression. The odds ratio suggests that with a TILs percentage of ≥20 %, the likelihood of positive PD-L1 expression in the clone SP142 is 375.5 times higher than when the TILs percentage is <20 %. Similarly, when the TILs percentage is ≥60 %, the odds of PD-L1 positive expression in the clone 22C3 are 106.3 times greater than when the TILs percentage is below 60 %. Using these TILs percentage benchmarks helps us make more accurate decisions to perform PD-L1 immunohistochemical studies when there are more TILs above the defined cut-offs.

3.5. Tertiary lymphoid structures (TLS)

Our research also explored the relationship between PD-L1 expression status and the presence of tertiary lymphoid structures (TLSs) using two distinct PD-L1 clones: SP142 and 22C3 (Table 6). For the clone SP142, a pronounced association was observed: samples with TLSs predominantly tested positive for PD-L1 expression (18 positive vs. 4 negative). This implies that TLSs could be indicative of PD-L1 positivity for the clone SP142. In contrast, for the clone 22C3, the association was less distinct but still noteworthy: among the samples with TLSs, 9 were positive for PD-L1, while 13 were negative. Our findings indicates a higher prevalence of PD-L1 positivity in TLS-positive cases, especially notable in the SP142 clone. The data further revealed a connection between Tumor-Infiltrating Lymphocytes (TILs) percentages and the presence of TLSs (Table 7). Specifically, samples with higher TIL percentages (particularly 70 % or more) were more likely to contain TLSs. The interplay between higher TIL percentages and the occurrence of TLSs opens avenues for deeper investigations into their intertwined relationship. These insights could be pivotal for enhancing diagnostic methods and advancing our comprehension of tumor biology.

4. Discussion

Quantitative TILs play a crucial role in TNBC evaluation. Elevated TIL levels not only provide positive predictive and prognostic value, irrespective of leukocyte subpopulations [7], but they are also correlated with improved responses to chemotherapy and better survival outcomes [4,5,8-11,32]. We advocate for pathologists to report TILs as the “percentage of stromal TILs” for every TNBC resection case. This reporting

should follow the guidelines set by the International Immuno-Oncology Biomarker Working Group on Breast Cancer, accessible at https://www.tilsinbreastcancer.org/. Pathologists should select the most representative tumor block for this assessment. At present, no consensus in the reporting format such as categories or threshold is available. We would like to offer a suggestion regarding the reporting of the TILs percentage parameter. While expressing TILs as a single specific number offers simplicity, it can result in discrepancies across pathologists and even within different tumor blocks. While a singular value can aptly represent TILs on the lower or higher spectrum—higher values being indicative of a favorable prognostic marker—this methodology may fall short in terms of comprehensive utility. Instead, we propose that the percentage of stromal TILs be reported in categories: <20 %, 20–60 %, and >60 %. Such categorization is likely more reproducible than a single value. Tumors with PD-L1 percentages between 20 and 60 % present a strong likelihood of expressing PD-L1, detectable using the PD-L1 IHC clone SP142. It would be prudent to encourage the testing of these cases for PD-L1 expression by clone SP142. Furthermore, cases with PD-L1 $\geq$ 60 % are likely to express PD-L1 detectable by both PD-L1 22C3 and SP142. Testing with any of these clones is advisable for these cases. On the other hand, tumors with stromal TILs under 20 % have a low likelihood of expressing PD-L1 by any of the two clones. In these situations, the necessity of PD-L1 testing should be evaluated on a case-by-case basis, weighing its potential value. It is essential to acknowledge that the specific cut-off might vary across institutions, contingent upon the chosen PD-L1 clone and associated drug. For instance, establishments employing SP142 with Atezolizumab might find categorizations of <20 % and $\geq$20 % sufficient. Meanwhile, those utilizing 22C3 alongside Pembrolizumab may deem divisions of <60 % and $\geq$60 % more suitable.

A significant challenge with the current protocol recommendation is its labor-intensive nature. This is mainly because it requires evaluation of the entire tumor field within a section rather than focusing only on hotspots. In our study, we tried to address this by examining TILs solely at the infiltrative edge, avoiding the need to assess the entire tumor region. However, we found variations: in some cases, while the edge presented abundant TILs, intralesional regions only showcased sparse TILs and vice versa. This inconsistency can likely be attributed to tumor heterogeneity, frequently observed in TNBC. Moreover, while optimal cut-off values for both clones seemed consistent with the overall TILs percentage, the specificity of TILs exclusively at the infiltrative edge was inferior. This was further evidenced by the heightened false-positive rate associated with both clones when compared to the entire lesional TILs. In conclusion, our data suggests that basing evaluations on stromal TILs at the infiltrative edge alone might not fully capture the true representation of the entire lesional TILs.

Tertiary lymphoid structures (TLS) have been observed at the infiltrative edge of tumors, and their potential significance in relation to TILs and PD-L1 expression has been of interest [7,20,21]. Our findings align with previous observations, indicating a strong association between higher TIL percentages, especially those exceeding 70 %, and the presence of TLSs. Notably, our data revealed a significant correlation between the presence of TLSs and PD-L1 expression for the clone SP142, where samples with TLSs were predominantly positive for PD-L1. This suggests that, at least for the clone SP142, TLSs may serve as a predictive factor for PD-L1 positivity. However, the correlation was less pronounced for the clone 22C3. Given the variability based on the PD-L1 clones and the intertwined relationship of TLSs with TIL percentages, the inclusion of TLSs in diagnostic reports, based solely on their association with PD-L1, may remain optional. Further exploration of these insights may offer pivotal advancements in our understanding of tumor biology and diagnostic methods.

Many studies have analyzed the rates of concordance and discordance for the 22C3 and SP142 clones. Unlike in other cancer types, TNBC sees lower TC (tumor cell) scores and higher IC (tumor-infiltrating immune cell) scores [29,30]. Despite IC scores tending to be higher than TC scores in TNBC, in a previous study, 22C3 assay showed a higher positive rate than SP142 assay and exhibited better agreement [28]. Additionally, the 22C3 assay may function as a more indicative prognostic marker compared to SP142 [28,31]. In our examination of 22C3 and SP142 across 141 cases, the concordance of 22C3 surpassed that of SP142, aligning with previous studies. However, differing from previous studies in our cohort of 141 TNBC cases, while the 22C3 clone detected PD-L1 expression in 14.9 % of cases, the SP142 clone identified a significantly higher rate of 50.4 %. This discrepancy implies that the clone 22C3 failed to detect 50 cases which were marked positive by SP142. On the contrary, no cases identified as positive by 22C3 were missed by SP142. While SP142 exhibits greater sensitivity than 22C3 in our study, each clone corresponds to specific FDA-approved therapeutic agents. As such, the two cannot be used interchangeably, emphasizing the critical clinical considerations in selecting the appropriate assay for TNBC patients.

Our study offers valuable insights into TNBC evaluation but is not without limitations. Notably, our data is from a single institute, possibly limiting its generalizability. While the TILs evaluation protocol is reproducible, it remains time-intensive, and subjectivity exists. As we look ahead, digital pathology stands out as a promising aid. Traditional histological assessments are intricate, but digital tools could streamline these processes, offering more efficiency. However, transitioning to such tools demands careful validation due to the intricacies involved.

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Consent for publication

All authors have provided consent for publication.

CRediT authorship contribution statement

Krit Suwannaphoom: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sithapong Soontornsit:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation, Conceptualization. **Kulachet Wiwatwarayos:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Prapapun Seneetuntigul:** Validation, Resources, Investigation, Formal analysis. **Parsinee Julimasart:** Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anndiagpath.2024.152294.

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